

THERMAL INACTIVATION STUDIES WITH VARIOLA VIRUS

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Received for publication October 3, 1960

In recent years, considerable interest has been manifested in the thermal inactivation of animal viruses (Friedman and De Berry, 1959; Lehmann-Grube and Syvertson, 1959; Youngner, 1957; Woese, 1960). Apart from the practical aspects, studies of the temperature sensitivity of virus particles offer a natural step in the analysis of their physicochemical properties (Gard and Maaløe, 1959) and, as such, may enhance our knowledge of the component parts of virus, namely, protein and nucleic acid.

The reaction characterizing the thermal inactivation of animal viruses usually follows first-order kinetics, but in some cases it has been found that the inactivation process proceeds in a two-component fashion (Bachrach et al., 1957; Kaplan, 1958). The characteristic features of animal virus thermal inactivations has been summarized by Woese (1960); the kinetics of the process at low temperatures involve one component; at high temperatures two components are manifested, each of first-order, with different values for the heat of activation of the processes. Studies with vaccinia virus, however, indicate that the shape of the inactivation curve at high temperatures may be of either one or two components, depending on the nature of the virus preparation or condition of virus storage prior to heating (Woodroffe, 1960). It appears, therefore, that the complexity of thermal inactivation of animal viruses is compounded further by certain pretreatment conditions of the virus.

Although variola virus is antigenically related to vaccinia virus, and may be assayed by a comparable, quantitative method (the inoculation of the chorioallantoic membrane of embryonated eggs), little is known of the reaction of variola virus to heat inactivation.

This report describes an attempt to characterize the thermal inactivation of variola virus and to determine the influence of certain factors on the reaction, i.e., suspending media, dilution of virus, and condition of virus storage.

MATERIALS AND METHODS

Virus preparation. The Yamada strain, as representative of variola virus, was used in the form of 20% chorioallantoic membrane suspension of the sixth egg passage. The history of the strain and the procedure employed for preparation of virus pools have been described previously (Hahon, Ratner, and Kozikowski, 1958). Samples of virus suspension were stored at -60°C in an electrically operated freezer.

Virus inactivation procedure. Depending on the nature of the experiment, vials containing 10 ml of either phosphate buffered saline pH 7.4, heart infusion broth (Difco) pH 7.4, 10% skim milk (Difco) pH 7.4, or 0.85% saline, pH 4.5, were warmed to the desired temperature and then seeded with 0.1 ml of an appropriate dilution of virus. The seeded vials were immersed in a water bath capable of holding the temperature constant to within $\pm 0.2^{\circ}\text{C}$. Immediately thereafter, 1.0 ml of suspension was removed from each vial; this served as a 0-min sample. Generally, at designated intervals of 15 min, a 1.0-ml sample was withdrawn from a vial and placed in an ice bath for subsequent assay.

Virus assay procedure. At the termination of an experiment, the viral content of samples was assayed as follows: Serial 10-fold dilutions ranging from 10^{-1} to 10^{-5} were made in heart infusion broth which contained 500 units of penicillin and 100 μg of streptomycin per ml. Undiluted samples or appropriate dilutions in 0.1-ml volumes were inoculated on the chorioallantoic membrane of 11- to 12-day embryonated eggs which had been prepared earlier for this route of injection (Hahon, Louie, and Ratner, 1957). Eight to 10 eggs were inoculated per dilution and incubated at 35°C for 72 hr. Harvested membranes were examined for pocks, and the number of infectious units per ml of test sample was calculated from the pock counts (Hahon et al., 1958).

RESULTS

Rates of inactivation of virus at different temperatures. Each of 8 vials containing 10 ml of warmed saline, pH 4.5, were seeded with 0.1 ml of a 10^{-2} dilution of variola virus. Pairs of vials were immersed in water baths of different temperatures, and samples were withdrawn and assayed as described previously. The effect of different temperatures on the thermal inactivation of variola virus is shown in Fig. 1. Within the limits of assay and replication, the data fit straight lines, indicating that the destruction of virus is exponential with the rate of the reaction dependent on temperature. Accordingly, the specific reaction rates were calculated from the equation for a first-order reaction

$$\frac{V_t}{V_0} = e^{-k_2 t}$$

in which V_0 is concentration of active virus at time zero, V_t is concentration of active virus at time t (minutes of incubation at given tempera-

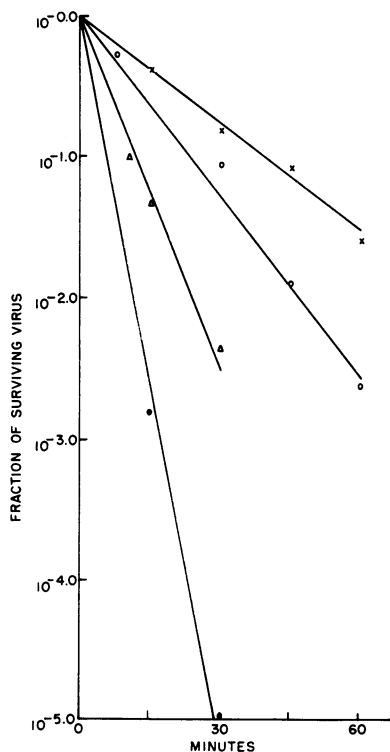


Fig. 1 Thermal inactivation of variola virus at different temperatures in saline pH 4.5; (X) 40 C, (O) 45 C, (Δ) 50 C, and (\bullet) 55 C.

TABLE 1

Reaction rate (k) and virus half-life ($t_{1/2}$) for the inactivation of variola virus at different temperatures in saline, pH 4.5

Temp	K	$t_{1/2}$
C	min^{-1}	min
40	5.7×10^{-2}	12.1
45	9.6×10^{-2}	7.1
50	1.8×10^{-1}	3.8
55	3.5×10^{-1}	2.0

ture, C), and K_2 is the rate of inactivation of virus at given temperature, C (min^{-1}). The reaction rates are given in Table 1.

The reaction rate as a function of the absolute temperature is expressed according to the Eyring theory of absolute reaction rates (Glasstone, Laidler, and Eyring, 1941). The Eyring equation, relating the reaction rate constant, k , to thermodynamic parameters is

$$k = \frac{KT}{h} e^{-\frac{\Delta H}{RT}} e^{\frac{\Delta S}{R}}$$

where K is Boltzmann's constant, T is absolute temperature, h is Planck's constant, R is the gas constant, and ΔH and ΔS are, respectively, the heat and entropy of activation for the process.

As shown in Fig. 2, ΔH may be obtained by the Arrhenius plot in which the natural logarithm of the k values ($\log_e k$), derived from the data in Fig. 1, is plotted against the reciprocal of the absolute temperature ($1/T$). A straight line is formed of which the slope $-\Delta H/R$ provides a value for ΔH of 28,000 calories per mole. A large positive value of this order has been noted for protein denaturation, a fact which suggests that the thermal inactivation of variola virus results from denaturation of virus proteins. By appropriate substitution in the equation for absolute reaction rates, an entropy (ΔS) value of 25.1 calories per mole per C was obtained. In the inactivation of enzymes and the denaturation of proteins, it is common to find values of ΔS of the order of 10 to 100 calories per mole per degree (Pollard, 1953).

The half-life ($t_{1/2}$) of the virus at each temperature may be determined either by inspection of Fig. 1 or by substitution in the equation

$$t_{1/2} = \frac{0.69}{k}$$

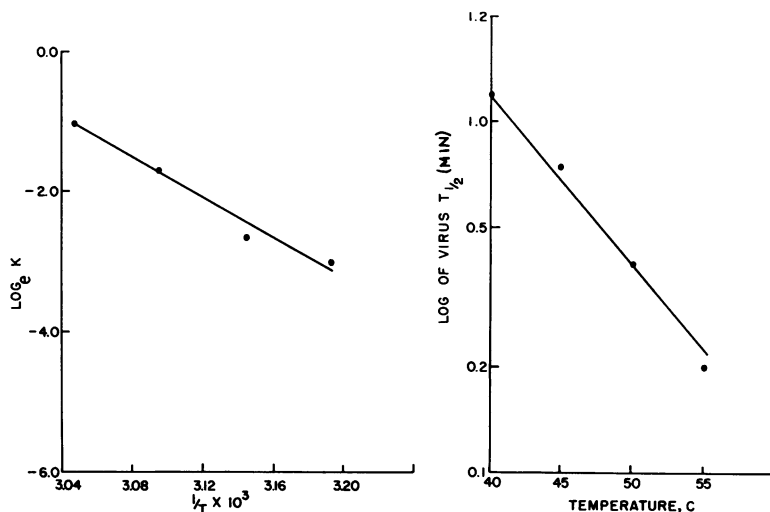


Fig. 2 (left). Relationship between the specific reaction rate for the inactivation of variola virus and absolute temperature according to the Arrhenius plot.

Fig. 3 (right). Relationship between the half-life ($T_{1/2}$) of variola virus and temperature.

in which k is the rate of reaction at a given temperature condition. Values of $t_{1/2}$ for variola virus at different temperatures are given in Table 1. It is evident that increases in the reaction rates result in decreases of the virus half-life. From the data, it is possible to interpolate the half-life at temperatures between 40 and 55 C when the logarithm of the $t_{1/2}$ values in Table 1 are plotted against temperature (Fig. 3). A reasonable straight line is formed from which the half-life of the virus may be determined within the intervening temperatures.

Influence of suspending fluid on inactivation curves. The results in Fig. 4 reveal that the inactivation of variola virus is affected by the media in which it is suspended for testing. The inactivation process appeared to be linear with all three suspending media, but the rates of the reaction were different. Skim milk, heart infusion broth, and phosphate buffered saline, in the order of listing, showed the greatest capacity to protect the virus from the effects of heat.

Different dilutions of virus and reaction rate. It has been observed that the initial concentration of the hemagglutinin of influenza virus affected the reaction rates (Miller, 1944; Lauffer and Carnelly, 1945); the rate of inactivation varied inversely with initial concentration. Accordingly, the effect of different initial dilutions of variola

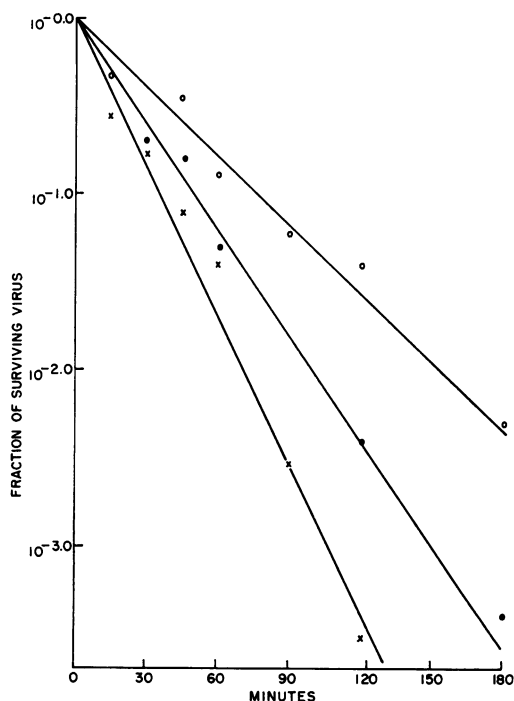


Fig. 4. Reaction rates of variola virus at 50 C in different suspending fluids of pH 7.4; (O) 10% skim milk, (●) heart infusion broth, and (X) phosphate buffered saline.

TABLE 2
Reaction rate (*k*) for the inactivation of variola virus
of different initial dilutions at 45 C
in saline, pH 4.5

Initial Dilution	Virus Concn <i>I</i> u/ml*	<i>k</i>
10 ⁻²	5.0 × 10 ⁴	2.0 × 10 ⁻²
10 ⁻³	5.0 × 10 ³	2.5 × 10 ⁻²
10 ⁻⁴	5.0 × 10 ²	5.7 × 10 ⁻²

* Infectious units.

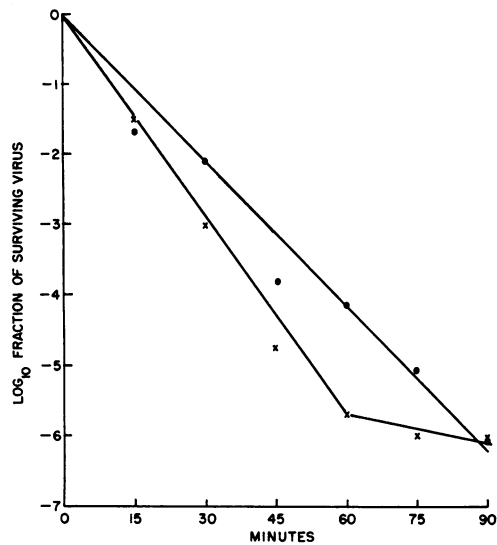


Fig. 5. Effect of storage at -60 C (●) and 4 C (×) on the inactivation curve of variola virus at 56 C in phosphate buffered saline (pH 7.4).

virus on the reaction rate was determined. The results in Table 2 indicate that the reaction rate was higher with dilute suspensions and lower with more concentrated suspensions of virus.

Effect of storage of virus on inactivation curve. At temperatures between 50 C and 60 C, the heat inactivation of an antigenically related pox-virus (vaccinia) was reported to involve a two component reaction (Kaplan, 1958). Further investigation of this phenomenon suggested that the reaction was dependent on the storage condition of preparations of vaccinia virus; prior to heating, virus stored at 4 C or -60 C was inactivated in a two component or one component manner, respectively (Woodroofe, 1960).

The possibility that different storage condi-

tions may affect, similarly, the inactivation curve of variola virus was determined by subjecting a preparation of virus that had been stored at -60 C and one that had been held at 4 C for 1 week to a temperature of 56 C. Both virus preparations were diluted 1:10 for testing. Results depicted in Fig. 5 were similar to those described by Woodroofe (1960) for vaccinia virus; a first-order reaction was noted with variola virus stored at -60 C; a two component curve was found with virus stored at 4 C.

DISCUSSION

The data presented in this report indicate that the thermal inactivation of variola virus between the temperatures of 40 C and 55 C follow first-order kinetics. Rates of the reaction were influenced by the suspending media and appeared to be affected by the initial dilution of virus. Although the effect of the latter was in agreement with studies cited previously regarding the effect of initial concentrations of the hemagglutinin of influenza virus on the inactivation rate (Miller, 1944; Lauffer and Carnelly, 1945), the possibility should be considered that a loss of native egg protein, by dilution of the virus suspension, may be a contributing factor affecting the reaction rate. This aspect of the experiment was not investigated further.

Although the shape of the reaction curve was independent of the range of temperature employed (Figs. 1 and 4), it appeared to be dependent on the condition of virus storage prior to heating (Fig. 5). Inactivation curves obtained with a suspension of variola virus stored at -60 C and a suspension of virus held at 4 C for 1 week and then subjected to 56 C gave results similar to those described by Woodroofe (1960) with vaccinia virus; a first-order reaction was noted with virus stored at -60 C, whereas a two component inactivation curve was obtained with virus stored at 4 C. Additional findings with vaccinia virus indicated that the inactivation curve was affected by the concentration of stored virus; a diluted suspension of virus stored at 4 C was inactivated at 50 C in a one component manner, but concentrated virus held at 4 C and diluted prior to heating exhibited a two component curve (Woodroofe, 1960). This suggests that during storage of concentrated suspensions of virus in the liquid state, some physical or chemical change occurred which greatly in-

creased the heat stability of a small proportion of the viral particles. Obviously, this explanation does not account for the nature of the mechanism by which stored viral preparations are affected and may not serve to explain the fact that two component inactivation curves were noted with poliovirus (Youngner, 1957) and with foot-and-mouth disease virus (Bachrach et al., 1957) which had been stored before heating at -20 and -40 C, respectively.

The ΔH value of 28,000 for variola virus reported in this study compared favorably with the range of ΔH of 20,000 to 90,000 for vaccinia virus, an antigenically related poxvirus (Kaplan, 1958). The meaning of the thermodynamic quantities ΔH and ΔS is questionable when dealing with large and complex units like virus particles. The difficulties and pitfalls encountered in trying to interpret ΔH and ΔS have been reviewed by Pollard (1953). In this report, the meaning of these quantities serve as constants characterizing the thermal inactivation reaction.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of James J. Friel and Lee E. Carey in certain phases of the work.

SUMMARY

Varied reaction rates at different temperatures (40 to 55 C) were demonstrated for the thermal inactivation of variola virus which were found to follow first-order kinetics. The rate of reaction was affected by different suspending media and appeared to be influenced by the initial dilution of virus. Storage of virus prior to heating at 56 C was found to affect the inactivation curve; a first-order reaction was noted with virus stored at -60 C, but a two component curve was observed with virus stored at 4 C for 1 week.

The heat of activation (ΔH) and entropy (ΔS) were 28,000 calories per mole and 25.1 calories per mole per C, respectively.

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